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**(64)** **Stable erythropoietin preparation and process for formulating the same.**

**(57)** A stable erythropoietin preparation and a process for formulating the same are disclosed.

Erythropoietin is useful in the treatment or diagnosis of anemia, but it is an instable substance. In order to provide a stable erythropoietin preparation, it is formulated with one or more stabilizers selected from the group consisting of polyethylene glycols, proteins, sugars, amino acids, inorganic salts, organic salts and sulfur-containing reducing agents.

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STABLE ERYTHROPOIETIN PREPARATION AND  
PROCESS FOR FORMULATING THE SAME

The present invention relates to a stable erythro-  
5 poietin preparation and a process for formulating the same.

Erythropoietin is a circulating glycoprotein that  
stimulates the formation of red blood cells and is useful  
in the treatment or diagnosis of anemia. A single dose of  
erythropoietin is as small as a few micrograms and this  
10 level must be strictly observed. In other words, erythro-  
poietin must be administered in the accurately measured trace  
amount in which it is formulated in a dosage form. However,  
erythropoietin is an instable substance and the compound  
purified to a clinically acceptable level and formulated in  
15 a trace amount ( $\approx$  a few  $\mu$ g) that is suitable for a single  
dosage will be easily inactivated by such environmental  
factors as temperature and humidity. It is therefore  
desired to formulate erythropoietin in a stabilized form.

As a result of various studies made in order to  
20 formulate a stable erythropoietin preparation, the present  
inventors found that this object can be attained by mixing  
erythropoietin with a particular compound. The present  
invention has been accomplished on the basis of this finding.

25 In accordance with one aspect of the present inven-  
tion, an erythropoietin preparation is provided that  
contains one or more stabilizers selected from the group  
consisting of polyethylene glycol, protein, sugar, amino  
acid, inorganic salt, organic salt and sulfur-containing  
30 reducing agent.

In accordance with another aspect of the invention, a  
process is provided for formulating a stable erythropoietin  
preparation containing one or more of the stabilizers listed  
above,

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The only requirement for the erythropoietin prepara-  
tion in accordance with one aspect of the present invention  
is that at least one of the stabilizers listed above be

mixed with erythropoietin. This erythropoietin preparation may be formulated in any dosage form such as a liquid dosage form, a solid dosage form (e.g. tablet, capsule, fine granule, granule or powder), or semisolid dosage form  
5 (e.g. suppository).

In accordance with the present invention, the erythropoietin may be obtained by any known method; it may be extracted from human urine, followed by separation and purification; alternatively it may be produced in E. coli,  
10 yeasts or Chinese hamsters ovary cells by genetic engineering technology, and extracted from the culture by a variety of methods, followed by separation and purification. The dosage of erythropoietin varies with the object of a specific diagnosis or treatment, and formulations containing 0.1 -  
15 50  $\mu$ g of erythropoietin per unit dosage form may be used. It should however be noted that the present invention is not limited by the erythropoietin content.

Proteins used as erythropoietin stabilizers include bovine serum albumin and gelatin; sugars include mono-  
20 saccharides such as xylose, mannose, glucose and fructose, disaccharides such as lactose, maltose and sucrose, trisaccharides such as raffinose, polysaccharides such as dextran, sugar alcohols such as mannitol, sorbitol and glycerol, and cyclitols such as inositol; amino acids  
25 include glycine, alanine and lysine; inorganic salts include potassium chloride, calcium chloride, sodium phosphate, potassium phosphate and sodium hydrogencarbonate; organic salts include sodium citrate, potassium citrate and sodium acetate; and sulfur-containing reducing agents include  
30 glutathione, thiocetic acid, sodium thioglycolate, thio-glycerol,  $\alpha$ -monothioglycerol and sodium thiosulfate.

These stabilizers are preferably incorporated in amounts of 0.1 - 10,000 parts by weight per part by weight of erythropoietin. Two or more of these stabilizers may  
35 be used if their total amount is within this range.

Erythropoietin may be administered by various routes such as injection (e.g. iv or mv), oral administration into digestive tracts, and transmucous administration directed

to the mucus of the rectum, oral cavity or nasal cavity.

Dosage forms suitable for the administration of erythropoietin include a liquid dosage form (e.g. injection), a solid dosage form (e.g. tablet, capsule, fine granule, granule or powder), and semisolid dosage form (e.g. suppository). Any conventional pharmaceutical additive may be mixed with erythropoietin as well as the stabilizers falling within the scope of the present invention so long as its stability is not impaired.

10 In order to prepare an erythropoietin injection, the stabilizer is used in the form of an aqueous solution having the proper concentration and pH. The osmotic pressure ratio of this aqueous solution is generally in the range of 0.1 - 3.0, preferably 1.0. The pH of the aqueous solution is  
15 generally adjusted to be within the range of 5.0 - 9.0, preferably 6.0 - 8.0. An anti-adsorption agent is preferably used in formulating the erythropoietin preparation of the present invention in the form of a solution such as injection, or when handling erythropoietin in the liquid  
20 state during formulation.

The following Experiments and Examples are provided for the purpose of further illustrating the present invention.

#### Experiments

25 (1) Erythropoietin derived from human urine (0.1 mg) was dissolved in 200 ml of 0.01% lecithin suspension and 5-ml portions of the solution were charged into test tubes. The stabilizers shown in the following table were put into the test tubes in the amounts also indicated in the table. Each  
30 of the mixtures was distributed among 10 vials in amounts of 0.5 ml and freeze-dried. The 10 vials were divided into two groups, each consisting of 5 vials. The freeze-dried mixtures of one group were immediately dissolved in an aqueous solution containing 0.1% bovine serum albumin, 0.15M NaCl  
35 and 0.01M  $\text{CaCl}_2$ , and the mixtures of the other group were left to stand at 37°C for one month before they were dissolved in the same solution. The activity of erythropoietin in each group was measured by the fasted rat method

described in Goldwasser, E. & Gross, M., Methods in Enzymol.,  
vol. 37, pp. 109 - 121 (1975). The percentage of residual  
activity was determined, with the value for the first group  
(dissolved in aqueous solution immediately after freeze-  
5 drying) being taken as 100. The results are shown in the  
following table in the column of "Percentage of residual  
activity - Freeze-dried". Each of the figures in the column  
was a mean of five measurements. The data in the table show  
the effectiveness of the stabilizing agents specified by the  
10 present invention.

(2) Erythropoietin derived from human urine (0.1 mg) was  
dissolved in 200 ml of 0.01% lecithin suspension and 5-ml  
portions of the solution were charged into test tubes. The  
stabilizers shown in the following table were put into the  
15 test tubes in the amounts also indicated in the table. Each  
of the mixtures was distributed among 10 vials in amounts  
of 0.5 ml. The vials were divided into two groups, each  
consisting of 5 vials. The activity of erythropoietin in  
the first group was immediately measured by the same fasted  
20 rat method that was used in Experiment (1), whereas the  
activity of erythropoietin in the second group was measured  
by the same method after standing at 25°C for one week.  
The percentage of residual activity was determined, with  
the average value for the first group being taken as 100.  
25 The results are shown in the following table in the column  
of "Percentage of residual activity - Aqueous solution".  
The data show that the stabilizers in accordance with the  
present invention are also effective in stabilizing the  
activity of erythropoietin in an aqueous solution.

30

Table

Stabilizer	Amount of stabilizer per part by weight of erythropoietin	Percentage of residual activity	
		Freeze-dried	Aqueous solution
Polyethylene glycol 4000	50	94	71
Gelatin	100	96	73
Dextran 40	50	98	70
Glycerol	100	95	67
Mannitol	100	95	69
Sorbitol	100	93	62
Inositol	100	98	71
Glucose	100	90	64
Fructose	100	93	65
Xylose	100	93	68
Mannose	100	99	71
Maltose	100	97	76
Sucrose	100	96	70
Raffinose	100	91	66
Glycine	100	96	75
Alanine	100	98	77
Lysine	100	88	63
NaCl	100	92	61
KCl	100	89	71
CaCl <sub>2</sub>	100	99	67
Potassium dihydrogenphosphate	36.2	92	67
Disodium hydrogenphosphate · 2H <sub>2</sub> O	71.4		

Table (cont'd)

Stabilizer	Amount of stabilizer per part by weight of erythropoietin	Percentage of residual activity	
		Freeze-dried	Aqueous solution
Potassium dihydrogenphosphate	28.8	-	69
Sodium hydrogencarbonate	69.9		
Acetic acid	0.6	-	69
Sodium acetate·3H <sub>2</sub> O	87.0		
Sodium dihydrogenphosphate·2H <sub>2</sub> O	97.7	87	70
Citric acid·2H <sub>2</sub> O	12.3		
Glutathione	10	90	66
Thioctic acid	10	90	67
Na salt of thioglycolic acid	10	89	68
Thioglycerol	10	87	65
α-monothioglycerol	10	89	67
Na salt of thiosulfuric acid	10	88	68
None	0	60	46

Example 1

Erythropoietin                      1 part by weight  
 Gelatin                                100 parts by weight  
 Distilled water for injection to make    10<sup>5</sup> parts by weight

5            An aqueous solution having the above composition was aseptically prepared, distributed among vials and freeze-dried, followed by the hermetic sealing of the vials.

Example 2

10           A freeze-dried erythropoietin preparation was formulated as in Example 1 except that 100 parts by weight of gelatin was replaced by an equal amount of dextran 40.

Example 3

A freeze-dried erythropoietin preparation was formulated as in Example 1 except that 100 parts by weight of gelatin was replaced by 500 parts by weight of polyethylene glycol 4000.

Example 4

	Erythropoietin	1 part by weight
	Human serum albumin	50 parts by weight
	Glycine	200 parts by weight
10	Mannitol	500 parts by weight
	Distilled water for injection to make	10 <sup>5</sup> parts by weight

An aqueous solution having the above composition was aseptically prepared, distributed among vials and freeze-dried, followed by the hermetic sealing of the vials.

15 Example 5

A freeze-dried erythropoietin preparation was formulated as in Example 4 except that the glycine and mannitol were replaced by 200 parts by weight of gelatin and 500 parts by weight of CaCl<sub>2</sub>.

20 Example 6

	Erythropoietin	1 part by weight
	Glutathione	10 parts by weight
	Human serum albumin	50 parts by weight
	Glucose	500 parts by weight
25	Distilled water for injection to make	10 <sup>5</sup> parts by weight

An aqueous solution having the above composition was aseptically prepared, distributed among vials and freeze-dried, followed by the hermetic sealing of the vials.

Example 7

30 A freeze-dried erythropoietin preparation was formulated as in Example 6 except that the glutathione and glucose were replaced by 10 parts by weight of thioglycerol and 500 parts by weight of maltose.

Example 8

35	Erythoropoietin	1 part by weight
	Dextran 40	50 parts by weight
	NaCl	500 parts by weight



Disodium	140 parts by weight
hydrogenphosphate·2H <sub>2</sub> O	
Potassium dihydrogenphosphate	70 parts by weight
Distilled water for	10 <sup>5</sup> parts by weight
injection to make	

An aqueous solution having the above composition was  
 5 aseptically prepared and distributed among ampules which  
 were then sealed by fusing.

Example 9

An ampule solution was prepared as in Example 8  
 except that the disodium hydrogenphosphate, potassium di-  
 10 hydrogenphosphate and NaCl were replaced by 98 parts by  
 weight of sodium dihydrogenphosphate·2H<sub>2</sub>O, 12 parts by  
 weight of citric acid monohydrate and 500 parts by weight  
 of sucrose.

Example 10

15 Nineteen parts by weight of Novata E (suppository  
 base of Henkel, Inc.) was dissolved in water at 40 - 60°C,  
 followed by gradual cooling. When the temperature of the  
 solution reached 40°C, it was mixed with one part by weight  
 of a separately prepared aqueous solution of erythropoietin  
 20 (containing 0.001 wt% of erythropoietin, 0.5 wt% of poly-  
 ethylene glycol 4000 and 5 wt% of Nikkol BL9EX, or a  
 surfactant produced by Nikko Chemicals K.K.) and an intimate  
 mixture was obtained under agitation. The resulting mix-  
 ture was poured into containers in amounts of about 2 g and  
 25 cooled to solidify as suppositories.

Example 11

A mixture of mannitol (5,000 parts by weight),  
 erythropoietin (1 part by weight), human serum albumin  
 (100 parts by weight), sodium acetyltryptophanate (2.154  
 30 parts by weight) and sodium caprylate (1.33 parts by weight)  
 was dissolved in purified water to make a total of 10<sup>5</sup> parts  
 by weight, and the resulting aqueous solution was freeze-  
 dried. The freeze-dried product was charged into capsules  
 specified in the Japanese Pharmacopoeia, which were covered  
 35 with an enteric coating agent by a known method, so as to  
 make enteric capsules.

Claims:

1. An erythropoietin preparation containing one or more stabilizers selected from the group consisting of polyethylene glycol, protein, sugar, amino acid, inorganic salt, organic salt and a sulfur-containing reducing agent.
2. An erythropoietin preparation according to Claim 1 wherein said protein used as erythropoietin stabilizer is bovine serum albumin or gelatin.
3. An erythropoietin preparation according to Claim 1 wherein said sugar used as erythropoietin stabilizer is any one of xylose, mannose, glucose, fructose, lactose, maltose, sucrose, raffinose, dextran, mannitol, sorbitol, glycerol and inositol.
4. An erythropoietin preparation according to Claim 1 wherein said amino acid used as erythropoietin stabilizer is any one of glycine, alanine and lysine.
5. An erythropoietin preparation according to Claim 1 wherein said inorganic salt used as erythropoietin stabilizer is any one of potassium chloride, calcium chloride, sodium phosphate, potassium phosphate and sodium hydrogencarbonate.
6. An erythropoietin preparation according to Claim 1 wherein said organic salt used as erythropoietin stabilizer is a citrate or an acetate.
7. An erythropoietin preparation according to Claim 1 wherein said sulfur-containing reducing agent used as erythropoietin stabilizer is any one of glutathione, thiocetic acid, sodium thioglycolate, thioglycerol,  $\alpha$ -monothioglycerol and sodium thiosulfate.
8. A process for formulating an erythropoietin preparation by mixing erythropoietin with one or more stabilizers selected from the group consisting of polyethylene glycol, protein, sugar, amino acid, inorganic salt, organic salt and a sulfur-containing reducing agent.
9. A process according to Claim 8 wherein said protein used as erythropoietin stabilizer is albumin or gelatin.
10. A process according to Claim 8 wherein said sugar used as erythropoietin stabilizer is any one of xylose,

monnose, glucose, fructose, lactose, maltose, sucrose, raffinose, dextran, mannitol, sorbitol, glycerol and inositol.

11. A process according to Claim 8 wherein said amino  
5 acid used as erythropoietin stabilizer is any one of glycine, alanine and lysine.
12. A process according to Claim 8 wherein said inorganic  
salt used as erythropoietin stabilizer is any one of sodium  
chloride, potassium chloride, calcium chloride, sodium  
10 phosphate, potassium phosphate and sodium hydrogencarbonate.



(19)



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# EUROPEAN SEARCH REPORT

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EP 85 11 3199

## DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. 4)
X	US-A-3 865 801 (S. CHIBA et al.) * Claim 1; column 4, lines 15-47 * ---	1,8	A 61 K 37/24
X	FR-A-2 501 692 (AJINOMOTO CO., INC. et al.) * Claim 1; example 1 * ---	1,8	
E	EP-A-0 178 576 (CHUGAI SEIYAKU K.K.) * Claims 1,3 * -----	1,2,3,8 9,10	
The present search report has been drawn up for all claims			TECHNICAL FIELDS SEARCHED (Int. Cl.4)
			A 61 K
Place of search		Date of completion of the search	Examiner
THE HAGUE		16-11-1988	PEETERS J.C.
CATEGORY OF CITED DOCUMENTS			
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document			

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